# An Immunological Study of Complementary Fragments of β-Galactosidase†

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ABSTRACT: Antibodies directed against complementary peptide fragments of *Escherichia coli* β-galactosidase react strongly with the wild type enzyme, indicating great similarity between tertiary structure of the fragments and of the enzyme. The yield of *in vitro* complementation can be significantly increased by specific anti-β-galactosidase antibodies.

The enzyme β-galactosidase from *Escherichia coli* is a tetrameric molecule, made up of four identical polypeptide chains, each with a molecular weight of 135,000. Complementation between both point mutants and deletion mutants affecting different segments of the polypeptide is observed *in vivo* and has been studied rather extensively *in vitro* (Ullmann *et al.*, 1968; Ullmann and Monod, 1970). Of particular interest are the complementation effects obtained when extracts are mixed of two types of mutants, one of which cuts out the N-terminal section of the polypeptide, while the other contains either a point-mutant, or a frameshift, or a deletion within the C-terminal section. The molecular weight of the C-terminal polypeptide involved in such complementations has been estimated as being about 40,000, *i.e.*, less than one-third of the total length of the wild type. Complementation, as measured by reappearance of activity, has been proved in such cases to involve the reassociation, into a tetrameric structure, of four "acceptor" polypeptides with four "ω" polypeptides (Goldberg, 1970).

The remarkably high efficiency of this phenomenon and the stability of the "pseudo-wild-type" structure thus reconstructed *in vitro*, must be taken to reflect the fact that the two partners (ω and acceptor) present very high and highly specific affinity with respect to one another. This in turn suggests that the ω peptide may be able spontaneously and by itself, to fold-up into a tertiary globular structure closely approximating that of the corresponding segment of the complete polypeptide within the native enzyme. If this interpretation is correct, it would involve the conclusion that the tertiary structure of a long polypeptide such as that of β-galactosidase, may be built up not necessarily *in toto*, but rather through the simultaneous (or stepwise) coiling up of different segments of the chain, each corresponding to a virtually independent center of nucleation.

It had not been established, however, so far, that the ω peptide by itself (in the absence of acceptor) does indeed assume a tertiary structure closely approximating that of the corresponding segment in the wild type molecule. In order further to investigate this point, we have felt that the application of immunological techniques might provide the most sensitive method of approach. Such an approach appeared justified by the well-established fact that the antigenic (and immunogenic) properties of proteins are closely related to and dependent upon the three-dimensional structure (Benjanini *et al.*, 1972). A most elegant and precise illustration of this has been provided by the work of Sela *et al.* (Arnon and Sela, 1969; Arnon *et al.*, 1971; Maron *et al.*, 1971) on the immunology of lysozyme. These authors have shown that a small peptide fragment ("loop peptide", MW 2700) obtained either by proteolytic cleavage of the molecule, or synthetically, would elicit antibodies able to react electively with the homologous portion of the native molecule. Conversely, a specific fraction of antibodies directed against the native molecule was shown to react strongly with the peptide, provided, however, that the "native" conformation had been restored through reformation of the correct disulfide bridge.

In the present paper we report the results of a systematic comparison of the antigenic and immunogenic properties of ω and acceptor, respectively, with those of wild type molecule. In addition we describe some observations concerning the effects of antigalactosidase antibodies upon the efficiency of the complementation reaction itself.

## Materials and Methods

*Bacterial Strains.* All strains used in this work were already described (Ullmann *et al.*, 1968). Wild type β-galactosidase was obtained from strain 2E01c, *in vivo* ω-complemented β-galactosidase from strain U366/FB9; ω peptide from strains B 9, M 15, and W 4680 and acceptor from strains S9080 and A238. For certain experiments *Lac* deletion strain 3000X74 was used.

*Preparation of Extracts and Purification Procedures.* Crude extracts were prepared in either $PM_2$[1] buffer ($Na_2HPO_4$-$NaH_2PO_4$, $10^{-1}$ M, $MgSO_4$, $10^{-3}$ M, $MnSO_4$, $2 \times 10^{-4}$ M, Mg-titriplex, $2 \times 10^{-3}$ M, β-mercaptoethanol, $10^{-1}$ M (pH 7.0)) or Tris buffer (Tris $2 \times 10^{-2}$ M, $MgSO_4$ $10^{-2}$ M, pH 7) by sonic disintegration of the bacteria. Generally 1 g wet weight bacteria suspended in 1.5 ml of buffer

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[1] Abbreviations used are: TVNS buffer, $2 \times 10^{-2}$ M Tris, $10^{-2}$ M EDTA, $10^{-2}$ M NaCl, and $10^{-1}$ M β-mercaptoethanol (pH 7.2); $PM_2$ buffer, $10^{-1}$ M $Na_2HPO_4$-$NaH_2PO_4$, $10^{-3}$ M $MgSO_4$, $2 \times 10^{-4}$ M $MnSO_4$, $2 \times 10^{-3}$ magnesium titriplex, and $10^{-1}$ M β-mercaptoethanol (pH 7).

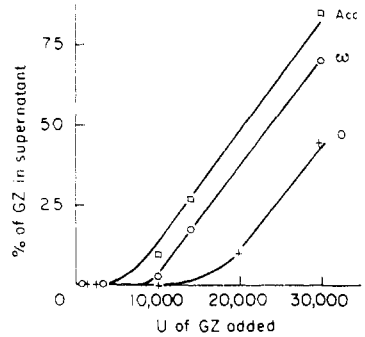


FIGURE 1: Effect of ω and acceptor on the saturation of anti-β-galactosidase antibody by β-galactosidase. To three series of tubes each containing 0.2 g of Sepharose-linked anti-β-galactosidase 0.2 ml of different extracts containing ~40 mg/ml of protein were added. After 2 hr of incubation at room temperature, the tubes were centrifuged and the supernatants discarded. Increasing amounts of pure β-galactosidase were added to each series of tubes which were further incubated for 18 hr (slow rotation in an inclined position). β-Galactosidase activity was assayed in the supernatants. The curves correspond to three types of extracts added to the Sepharose-linked antibodies: (+) Lac deletion (o); (O) ω; (□) acceptor (Acc); (GZ) β-galactosidase.

yields a crude extract containing approximately 40 mg/ml of protein.

Wild type β-galactosidase was purified to homogeneity according to known procedures. Its specific activity was 800,000 U/mg.

*In vitro* complemented enzyme was purified by the same techniques as the wild type one, with an additional step consisting of a sucrose gradient centrifugation. The specific activity of the purified complemented enzyme was 400,000 U/mg.

The final centrifugation step separated the complemented β-galactosidase (16 S) from the major contaminating material (6 S). The latter has been identified as free acceptor by the criteria of complementing activity and binding to anti-β-galactosidase antiserum.

Pure ω was obtained from *in vivo* complemented β-galactosidase. The purified enzyme dissolved in TVNS[1] buffer (Tris $2 \times 10^{-2}$ M, EDTA $10^{-2}$ M, NaCl $10^{-2}$ M, β-mercaptoethanol $10^{-1}$ M (pH 7.2)) was dialyzed against 8 M urea dissolved in the same buffer. The dissociated enzyme was chromatographed on a Sephadex G-100 column equilibrated with TVNS buffer. Pure ω was eluted as a unique peak. Its specific activity, determined by complementation, was of $1.4 \times 10^6$ U/mg of protein. Taking into account the specific activity of the complemented enzyme, the expected specific activity of ω would be $1.6 \times 10^6$ assuming 100% recovery of ω activity.

*In vitro Complementation.* If not otherwise specified, the complementation reaction was performed by mixing equal volumes of crude extracts of W4680 (ω donor) and S9080 (acceptor). After 90 min of incubation, the mixture was diluted 10–20 times and β-galactosidase activity measured.

*Immunization Procedures.* Rabbits (2–4) for each antigen were immunized with the following proteins: pure wild type β-galactosidase, purified acceptor, and ω obtained by the procedure described above. As a rule, two injections of 3 mg of antigen in Freund's adjuvant were given in the footpad at 1-month interval. For immunization with ω, the dose was reduced to 0.75 mg for each injection.

*Preparation and Purification of Fab Fragments.* Monovalent antibody fragments were produced by papain cleavage of immunoglobulins isolated from anti-β-galactosidase antiserum. The procedure is derived from Porter (1959) and has been described elsewhere (Celada *et al.*, 1970). The binding activity of Fab was determined by inhibition of β-galactosidase precipitation by antiserum.

TABLE I: Effect of ω and Acceptor on the Binding of Anti-β-galactosidase Fab to β-Galactosidase.

| Expt. No. | Total Anti-β-galactosidase Fab (cpm) | Addition | Cpm Eluted | Competition (%) |
|---|---|---|---|---|
| 1 | 4246 | None | 0 | 0 |
| 2 | 3380 | None | 0 | 0 |
| 1 | 4246 | ω | 1680 | 39 |
| 2 | 3380 | ω | 1280 | 38 |
| 1 | 4246 | Acceptor | 3261 | 77 |
| 2 | 3380 | Acceptor | 2249 | 67 |

[a] To 0.1 ml of Sepharose-linked β-galactosidase packed in microcolumns, $^{125}$I-labeled, purified (see Materials and Methods Section) Fab was added, in the absence or presence of extracts containing either ω or acceptor in saturating amounts. The columns were washed and the radioactivity was determined in the effluent. (The Fab preparation used in experiment 1 contained 6078 cpm. It was determined (data not shown) that 1838 cpm were unrelated to specific antibody. The data represent corrected values.)

Immunological purification of anti-β-galactosidase Fab was achieved by the following steps: (a) 10 mg of Fab protein was passed twice on a column packed with 4 g of Sepharose-linked β-galactosidase (the final effluent had only traces of Fab activity); (b) after extensive washing of the column with low ionic strength ($10^{-3}$ M) phosphate buffer at pH 7, the pH was lowered to 2.5 by addition of HCl, and 6 ml of effluent was collected and immediately neutralized with 2 ml of $10^{-1}$ M phosphate buffer (pH 7.0).

After concentration the protein was labeled with $^{125}$I by the standard chloramine T method and dialyzed. The final product had a specific activity of ~40,000 cpm/mg of protein. More than 70% of the label was associated with specifically binding Fab.

Covalent binding of antibodies and enzyme on Sephadex was performed using the method of Givol *et al.*, (1970). The attachment of β-galactosidase, ω, and acceptor to the activated Sepharose was performed in $PM_2$ buffer instead of using $NaHCO_3$.

## Results

*A. Immunological Activity of ω and Acceptor Fragments.* The antigenicity of ω and acceptor fragments (*i.e.*, their capacity to react with anti-β-galactosidase antibodies) was tested by measuring their capacity to compete with β-galactosidase in the binding of anti-β-galactosidase antibodies. Figure 1 shows the saturation profile of Sepharose-linked antibodies and the displacement of the saturation point by either of the two fragments. It can be seen that both ω and acceptor reduce considerably the saturation point, showing that antibody directed against wild type enzyme can bind each of the two peptide fragments (ω and acceptor).

This result has been confirmed using purified antigalactosidase monovalent Fab labeled with $^{125}$I and measuring its binding to either acceptor or ω (Table I). The maximal

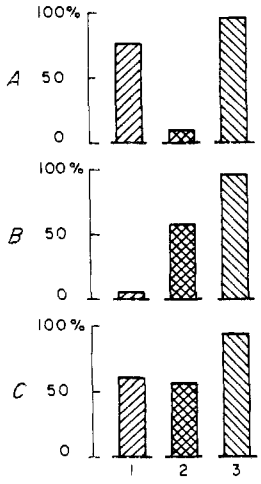


FIGURE 2: Specificity of anti-ω and antiacceptor antibodies. To 0.2 g (wet weight) of different Sepharose-linked antibodies, 0.4 ml of crude extracts containing ω, acceptor, or β-galactosidase in $PM_2$ buffer (~900 U/ml) was added. The mixtures were gently rotated in tubes at room temperature for 2 hr, then centrifuged. The complementing activities of ω and acceptor as well as the enzymatic activity of β-galactosidase were determined in the supernatants. The figure represents the retained fractions expressed as percentage of the total initial activity. (A) anti-ω Sepharose; (B) antiacceptor Sepharose; (C) anti-β-galactosidase Sepharose; (1) ω added; (2) acceptor added; (3) β-galactosidase added.

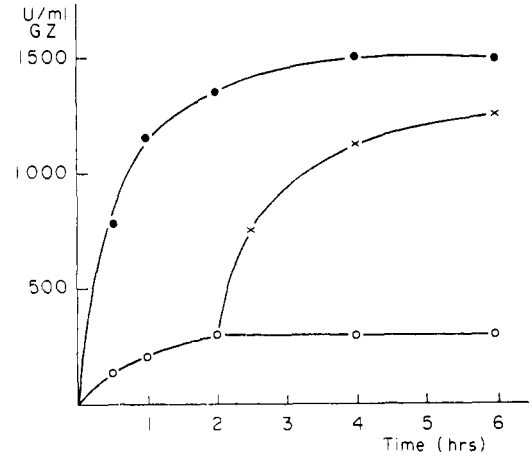


FIGURE 3: Effect of anti-β-galactosidase antibodies on complementation. Equal volumes of extracts containing acceptor (5 mg of protein/ml) and ω (30 mg of protein/ml) prepared in $PM_2$ buffer were mixed at time = 0. (○) no addition; (●) addition of anti-β-galactosidase antibody at $t$ = 0. (x) addition of anti-β-galactosidase antibody at $t$ = 2 hr; GZ, β-galactosidase.

capacity of the column was determined. If now a barely saturating amount of Fab is passed through such a column, all the label is retained. If this assay is repeated in the presence of increasing amounts of ω or acceptor, only part of the label is retained.

One could hardly fail to notice the fact that the percentage of competition observed with each of the two peptides corresponds roughly to their respective molecular weights. This may be taken to indicate that the large molecule of β-galactosidase presents a multiplicity of (different) antigenic determinants randomly distributed over its surface.

The immunogenicity of the ω and acceptor fragments was investigated. Antibodies directed against either one of the two fragments (purified by the procedure described in Materials and Methods) were obtained and their specificity was studied by comparing their relative capacity to bind wild type enzyme, ω, or acceptor. The experiments were performed using immobilized antisera. The concentrations of β-galactosidase, ω, and acceptor were chosen so as to have approximately the same amount of enzyme units (measured directly for β-galactosidase and under optimal complementation conditions for the two peptides). Assuming that the efficiency of complementation is the same for ω and acceptor, the binding of each protein to the different antisera can be roughly compared. The results are presented in Figure 2 and the following conclusions can be drawn: (1) antigalactosidase binds to about the same extent ω and acceptor; (2) anti-ω and antiacceptor bind almost exclusively to the homologous antigen; (3) both sera bind the wild type enzyme.

The slight heterologous binding can be accounted for by the overlap of the N-terminal sequence of ω with the C-terminal of the acceptor.

It may at first sight appear surprising that anti-ω should apparently bind more β-galactosidase than ω itself, the homologous antigen. Taking into account that the specific activity of the complemented enzyme is half that of the wild type one such a result should in fact be expected if the cross-reaction between β-galactosidase and ω is close to 100% in respect to antigenic determinants present on the ω fragment.

*B. Effect of Antisera on ω Complementation.* It appeared of interest to study possible effects of the different antisera upon the complementation reaction.

Figure 3 shows the effect of anti-β-galactosidase antiserum on complementation. It can be seen that antibody increases the yield of complementation without significantly altering the rate constant of the reaction which, under these conditions, appears to be roughly first order. Moreover the plateau values are similar whether the antibody is added at the time of mixing the extracts or after "completion" of the reaction in its absence.

The magnitude of the antibody effect is quite variable and depends on the relative concentrations of ω and acceptor. The maximal effect can be observed at low acceptor and high ω concentrations.

Figure 4 shows the plateau levels obtained in the absence or presence of antibody at different acceptor concentra-

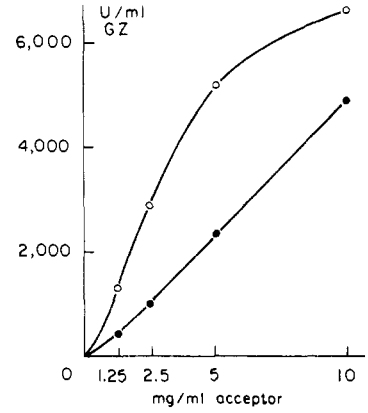


FIGURE 4: Effect of antibody on complementation mixtures containing increasing concentrations of acceptor. To 0.1 ml of an extract containing ω (40 mg of protein/ml) 0.1 ml of an extract containing acceptor at various protein concentrations (see values on the abscissa) was added. After 90 min of complementation, β-galactosidase activity was measured. (●) no addition; (○) addition at time = 0 of anti-β-galactosidase Fab at final protein concentration of 0.3 mg/ml. GZ, β-galactosidase.

TABLE II: Effect of Various Sera on ω Complementation.

| Addition | β-Galactosidase (U/ml) |
|---|---|
| None | 1,940 |
| Anti-β-galactosidase | 10,120 |
| Fab anti-β-galactosidase | 11,000 |
| Antiacceptor | 1,440 |
| Anti-ω | 2,080 |
| Anti-ω + antiacceptor | 1,900 |
| Normal rabbit serum | 1,520 |

[a] Complementation was performed as described in the Materials and Methods section.

tions. At saturating acceptor concentrations the effect of the antibody becomes negligible.

Table II shows the effect of different antisera on complementation. As it can be seen the yield of complementation is strongly increased in the presence of anti-β-galactosidase but anti-ω or antiacceptor antisera show no significant effect, whether they are added separately or mixed together. This means that anti-β-galactosidase serum contains antibodies which do not exist either in anti-ω or antiacceptor sera. It can also be seen in the table that enhancement of complementation does not require the action of bivalent antibodies since Fab fragments produced by papain digestion of anti-β-galactosidase antibodies acted in a way similar to intact antiserum.

## Discussion

The essential point of the experiments presented in this paper was to test the assumption that the C-terminal peptide fragment (ω) of the β-galactosidase polypeptide is able *by itself* (that is, in the absence of specific stabilizing interactions) to fold into a configuration closely approximating that of the corresponding segment in the wild type enzyme.

It may be worth pointing out first (for those readers who may have skipped through the Materials and Methods section), that the ω preparation used in immunization experiments was purified from *in vivo* complemented enzyme by a procedure involving complete denaturation of the protein in 8 M urea, followed by gel filtration in presence of buffer, during which the complementing activity of ω is restored with an efficiency close to 100%. This means that the ω fragment is able, not only *in vivo* but *in vitro* as well to fold independently into a configuration which cannot be very far (structurally and energetically) from that of the corresponding segment in the active wild type enzyme.

This is confirmed by the fact that antibodies prepared against the wild type enzyme bind efficiently both ω and the complementary ("acceptor") fragment. However, both in the complementation and in the antibody binding test, a specific stabilizing interaction is evidently involved, and it is therefore not excluded that *in the absence* of such interactions the wild type conformation does not actually obtain in the ω fraction.

By far the most significant finding therefore certainly is that both ω and acceptor, when used as *immunogens*, elicit the formation of antibody which binds avidly the wild type enzyme. This we believe may be taken as straightforward evidence that, in the absence of specific stabilizing interactions, the configurations which obtain in both the acceptor and ω fragments approximate closely the wild type folded structure. This is particularly significant in the case of ω, since as we pointed out above, this fraction had been denatured and renatured *in vitro* before being used as immunogen.

The effects of different antisera on the efficiency of complementation can be considered to further strengthen this conclusion. The fact that antigalactosidase (wild type) serum exerts a powerful enhancing effect is interpretable in terms of the presence within such sera, of antibody directed against antigenic determinants whose area overlap regions of contact between the complementary fractions. Another interpretation could be offered, however, namely that antibody directed against the wild type structure may be stabilizing a competent wild type configuration in either or both fragments. This interpretation would involve the assumption that the free fragments do not actually, or only partially assume the "competent" configuration. If, however, this were the case we would expect anti-ω and/or antiacceptor sera to favor the free, noncompetent or less competent structure in these fractions and therefore to *inhibit* complementation. Actually, no such effect is observed which can be taken to further confirm the conclusion that: (a) no stabilization of the wild type configuration in the complementary fractions is required to allow or enhance complementation; (b) the enhancing effect observed with anti wild type serum is indeed due mainly, if not exclusively, to "overlapping" antigenic determinants.

In summary, on the basis of these findings, we are led to conclude that the ω polypeptide, even though it corresponds to only 30% of the complete wild type polypeptide, is able *by itself* (*i.e.*, in the absence of any interactions with other segments or parts of the wild type molecule) to fold into the correct wild type structure, or one very close to it. It seems reasonable, on this basis, to assume that the normal *in vivo* mechanism of folding of the wild type protein involves the stepwise activation of several virtually independent centers of nucleation.

That such a mechanism may intervene in the folding of the peptide backbone of many if not most proteins is indeed strongly suggested by simple inspection of a number of three-dimensional structures established on the basis of X-ray crystallography (Philips, 1967, Wetlaufer, 1973).

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# Preparation and Characterization of Antibodies to Menadione†

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ABSTRACT: Antibodies specific for menadione (2-methyl-1,4-naphthoquinone, vitamin $K_3$) were raised in rabbits following immunization with menadione–protein complexes wherein the protein was covalently bound to menadione at the three position *via* thioether linkage. The antibodies precipitated with menadione bound similarly to a different carrier protein and they were isolated from antimenadione sera with menadione-bearing immunoadsorbants. The binding of univalent menadione–haptens by purified antibodies was studied by equilibrium dialysis. The average intrinsic association constants for the binding of menadione–butyrate by antibodies produced early after immunization and those obtained after secondary immunization were $1.1 \pm 0.3 \times 10^5$ and $2.6 \pm 0.6 \times 10^6$ $M^{-1}$, respectively. Heterogeneity of hapten-binding sites appeared to be greater in the late antisera. The tryptophan fluorescence of antimenadione antibodies was quenched by bound menadione but the degree of quenching was less than has been observed for some antidinitrophenyl antibody–ligand systems.

Antibody-like activity against menadione or vitamin $K_3$ (2-methyl-1,4-naphthoquinone), the aromatic nucleus of the K vitamins, has been observed with several myeloma proteins and with rabbit and guinea pig antibodies raised against various nitrophenyl groups (Eisen *et al.,* 1970; Rosenstein and Richards, 1972; Michaelides and Eisen, 1974).

Accordingly, we wanted to determine whether antibodies could be raised against menadione by conventional immunization procedures and to evaluate the binding affinities of these antibodies. This paper describes the preparation of menadione–protein antigens and menadione–haptens, and some properties of antimenadione antibodies.

## Materials and Methods

*Conjugation of Menadione to Proteins.* Menadione–proteins were prepared by coupling menadione to bovine γ-globulin, BγG,[1] or human serum albumin, HSA, *via* a thioether linkage wherein thiol groups previously bound covalently to the proteins reacted with menadione by nucleophilic substitution (Nickerson *et al.,* 1963).

Thiol groups were attached to the proteins with *N*-acetylhomocysteine thiolactone (Singer, 1964). In a representative preparation, 400 mg of protein in 6 ml of 0.02 M potassium carbonate (pH 10.7) was mixed with 500 mg of *N*-acetylhomocysteine thiolactone in 2 ml of water and held for 18–24 hr at 4°. The solution was then adjusted to pH 5.0 with 2.5 N HCl, chromatographed on Sephadex G-25 (1.5 cm × 30 cm) in water, and the thiol content of the eluted protein was measured (Ellman, 1959). The pooled fractions of thiolated protein were brought to pH 5 with acetate buffer (pH 5.0) (final concentration 0.02 M) and stirred with a saturating amount of menadione for 30–60 min in the dark at room temperature. After decanting from residual solid menadione, the solution was dialyzed exhaustively against phosphate–saline[1] to remove unreacted menadione.

Ultraviolet absorption spectra of menadione–proteins were obtained in a Cary 14 recording spectrophotometer. The number of menadione groups per protein molecule was calculated from the dry weight of the protein and from absorbancy at 335 nm, which was assumed to be due entirely to the menadione moiety with a molar extinction coefficient of 2200 (Rosenberg, 1945). (This value is the same for free and protein-bound menadione in phosphate–saline.) Dry weight was determined by exhaustive dialysis of the proteins against distilled water followed by heating under vacuum at 100° to constant weight.

The menadione content of the conjugated proteins was also determined by a modification of a method (Strauss *et al* 1963) wherein menadione undergoes base-catalyzed hydrolytic cleavage from the protein, yielding sulfhydryl protein and phthiocol. For this assay 2 ml of 0.2 N NaOH

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[1] Abbreviations: BγG, bovine γ-globulin; phosphate–saline, 0.15 M NaCl–0.01 M sodium phosphate (pH 7.4); menadione–caproate, menadione–*N*-acetylhomocysteine thiolactone–ε-aminocaproate; menadione–butyrate, menadione–*N*-acetylhomocysteine thiolactone–γ-aminobutyrate; HSA, human serum albumin.